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Research Note

Leakage of Intracellular UV Materials of High Hydrostatic Pressure–Injured *Escherichia coli* O157:H7 Strains in Tomato Juice†

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ABSTRACT

The behavior of high hydrostatic pressure–injured *Escherichia coli* O157:H7 cells (strain SEA13B88 and a strain from the June–July 1999 Oklahoma juice outbreak) in tomato juice (pH 4.1) and phosphate-buffered saline (PBS; pH 7.2) at final concentrations of 8.4 to 8.8 log CFU/ml, respectively, and treated at 400, 500, and 600 MPa for 40 min at 25 and 35°C with storage at 5 and 23°C for 1,800 min was investigated. Immediately after treatment and every 3 h for 24 h of storage, an aliquot (0.1 ml) was plated on Trypticase soy agar and sorbitol MacConkey agar to determine the percentage of injured population. Leakage of UV materials and possible recovery from injury were investigated. Pressure (600-MPa) treatment at 35°C for 40 min caused a higher percentage of bacterial injury than for 10 min of treatment. A higher percentage of injured population was found among the Oklahoma strain cells than among strain SEA13B88 cells, and differences in viability loss for bacterial strains were determined. The viability loss determined in PBS was 4.8 log for SEA13B88 cells and 5.2 log for Oklahoma cells, while losses of 5.4 and 5.7 log were determined in tomato juice for SEA13B88 and Oklahoma cells, respectively. The leakage of intracellular materials of injured Oklahoma cells was higher than that observed for SEA13B88 cells, but injured Oklahoma cells recovered faster in PBS. However, injured and healthy populations for both strains were below detection in tomato juice stored at 5°C for 1,440 min.

Contamination of juices with pathogenic microorganisms has caused numerous illnesses and some fatalities. From 1923 to 2000, consumption of contaminated fruit juices has been implicated in at least 28 foodborne illness outbreaks *(15)*. Eleven (almost 40%) of 28 outbreaks were associated with *Salmonella* serotypes. Eight (close to 30%) of 28 outbreaks were caused by enteropathogenic *Escherichia coli,* especially *E. coli* O157:H7. Although most bacteria cannot grow at low pH, *E. coli, Shigella,* and *Salmonella* serotypes can survive for several days or weeks in acidic foods *(14, 16, 25, 40)*. Thermal processing is used by the juice industry to inactivate foodborne pathogens; however, it impairs the characteristic flavor of juices. Food manufacturers and distributors are responding to consumers' demand for food products that are safe, fresh, and convenient *(7, 29)*. In order to meet consumers' demand for ''freshness,'' some foods may be improperly processed and/or contaminated with spoilage bacteria or human bacterial pathogens *(5, 17, 19, 27)*. Foodborne outbreaks involving *E. coli* O157:H7 in apple and orange juices *(8–10, 13)* have raised concerns about the safety of consuming unpasteurized fruit juices.

There is a need for alternative nonthermal processes for foods that require minimal heat processing *(12, 33)*. Several technologies for nonthermal processing have been commercialized including high hydrostatic pressure (HHP) *(12, 18, 20, 23, 34, 36, 42)*. A relationship between membrane damage and cell death of *E. coli* strains in nutrient broth has been reported *(30, 35)*. Also, variation in resistance of *E. coli* O157:H7 to HHP, mild heat, and other stress including antimicrobials (pediocin AcH and nisin) has been reported *(4, 21)*. The effect of HHP on the survival of bacterial populations in liquid foods or liquid buffered system has been reported *(3, 24)*. In these reports, inactivation and survival and/or recovery of *E. coli* populations were discussed but not the details of membrane damage that led to inactivation or recovery of injured cells. In our study, we investigated the behaviors of two strains of *E. coli* O157:H7 (strain SEA13B88 and a strain from the June and July 1999 Oklahoma juice outbreak, hereafter referred to as Oklahoma) in tomato juice (pH 4.1) and phosphate-buffered saline (PBS; pH 7.3) treated with HHP at 25 or 35°C. The ability of HHP-injured *E. coli* O157:H7 SEA13B88 and *E. coli* O157:H7 Oklahoma cells to recover and survive in tomato juice (pH 4.1) and PBS (pH 7.3) during storage at 5°C and room temperature (approximately 23°C) for up to 30 h was investigated. Similarly, leakage of UV materials including ATP from the damaged cells was estimated and

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used to correlate the relationship between pressure and treatment temperature that led to viability loss.

MATERIALS AND METHODS

Bacterial strains and preparation of inoculum. Enterohemorrhagic *E. coli* O157:H7 SEA13B88 and *E. coli* O157:H7 Oklahoma from the U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, culture collection were used in this study. Individual cell cultures were maintained on Trypticase soy agar (TSA) at 4°C. Prior to use, the cells were inoculated by loop in tryptic soy broth (TSB; Remel, Inc., Lenexa, KS) followed by incubation at 37°C for 16 to 18 h with shaking. A 0.1-ml aliquot of the stationary-phase bacteria was transferred to 20 ml of TSB and incubated at 37°C for 24 h. The overnight cell suspensions were centrifuged at 3,000 × *g* for 10 min at 5°C. *E. coli* O157:H7 SEA13B88 and *E. coli* O157:H7 Oklahoma cell pellets were each washed with an equal volume (20 ml) of sterile PBS (pH 7.2) solution. The bacterial cells in PBS were held at 5°C for <30 min until they were used to inoculate test samples.

HHP treatment of tomato juice. Pasteurized tomato juice (pH 4.1) from concentrate (Value plus, Wal-Mart brand) purchased from a local store was used for this study. Three 25-ml sterile test tubes containing 9 ml of tomato juice or the PBS solution were inoculated with cell suspensions (1 ml) of each *E. coli* O157:H7 strain to give a final concentration of 2.5×10^8 to 6.5×10^8 CFU/ml. Bacterial cells suspended in PBS solution were referred to as resting cells in this study. All samples were vortexed, and 2 ml of each was dispensed into individual sterile plastic bags (5.0 by 8.0 cm) (W. R. Grace & Co., Cedar Rapids, IA) and sealed using a Doughboy heat-sealer (Doughboy Packaging Machinery, Inc., New Richmond, WI). Each plastic bag was double-bagged (7.0 by 10.0 cm), sealed to prevent leakage, and then kept at 4°C prior to pressurization (<1 h). All samples were placed inside a batch hydrostatic pressurization unit (model 2 L, AVURE Technology, Kent, WA) chamber filled with deionized water. HHP was applied at 400, 500, and 600 MPa for up to 40 min at 25 and 35°C. The pressure increase during treatments averaged 240 MPa/min, the come-up time was 45 s, and the come-down time was 6 s. All samples were removed immediately after pressurization and placed in an ice bath to cool (<1 h). Inactivation of *E. coli* O157:H7 populations in treated tomato juice and PBS were estimated by plating 0.1 ml on nonselective TSA plate. Surviving populations and injured *E. coli* O157:H7 cells were determined immediately after treatments by plating on differential agar (selective and nonselective) plates as described below. All tests were performed in duplicate within 1 h of HHP treatment.

Determination of injured bacteria and viability loss. To determine the initial and final bacterial populations in untreated and HHP-treated PBS and tomato juice, 0.1 ml of individual samples was plated in duplicate on TSA and sorbitol MacConkey agar plates. Viability loss and sublethal injury resulting from the pressure treatments were determined using differential plating methods on nonselective (TSA) versus selective (sorbitol MacConkey agar) plates. The number of CFU per milliliter on nonselective and selective agar media was used to calculate the viability loss, which is defined as the differences in log CFU of bacteria per milliliter between control and treated samples *(26)*. Samples treated at different pressures (400, 500, and 600 MPa) and temperatures (25 and 35°C) for 5, 10, 15, 20, 25, 30, 35, and 40 min were plated (0.1 ml) on agar plates as stated above, and the percent injury was calculated using the following formula:

$$\left(1 - \frac{\text{colonies on selective agar}}{\text{counts on nonselective agar}}\right) \times 100$$

Behavior of injured cells in PBS and tomato juice during storage. Samples treated at all pressures and temperatures stated above for 25 min were stored at 5°C and room temperature (23°C) to monitor the behavior of the initial surviving HHP-treated cells for possible recovery of injured cells during storage for up to 1,800 min (30 h). Treated tomato juice and PBS were plated (0.1 ml) on both TSA and MacConkey agar plates at 0 (25), 360, 720, 1,080, 1,440, and 1,800 min of storage to determine the numbers of CFU. The population of cells determined at 0 (25 min) as stated above represents the numbers that survived the pressure treatment at each temperature tested for 25 min. Bacterial cells from untreated inoculated tomato juice and PBS similarly stored and plated on agar media were used as positive controls for each experiment. All agar plates were incubated at 36°C for 48 h.

Leakage of intracellular substances from bacteria. Extracellular UV materials and ATP in PBS samples were quantified to establish a baseline value before pressure treatments were applied. Any increase of these baseline values after treatment would be attributed to have come from the damaged or injured cells. Leakage of intracellular bacterial ATP as a function of membrane damage was determined using an ATP bioluminescent assay kit and ATP luminometer (DL Ready and TD-20/20 luminometer, Turner Design, Sunnyvale, CA). Assays of standard amounts of purified ATP were used to calculate ATP levels, and ATP concentrations in samples were expressed as log femtogram per milliliter. Possible inhibition of the luciferase reaction by any residues from the tomato juice or the PBS was corrected for by addition of known amounts of ATP standard to the reaction vial followed by addition of the luciferase enzyme *(38)*. Protein and nucleic acid materials that came from damaged or injured *E. coli* cells were quantified spectrophotometrically (DUR 530, Beckman Coulter, Fullerton, CA) at 260 and 280 nm as previously reported *(6, 37, 39, 41)*.

Data analysis. All experiments were repeated three times, and samples obtained at each sampling time were analyzed in duplicate. Data were subjected to analysis of variance with the Statistical Analysis System Program (SAS Institute, Cary, NC). Significant differences ($P < 0.05$) in survival, injury, inactivation, and leakage of ATP or intracellular UV materials from the injured bacteria were determined by the Bonferroni LSD method *(28)*.

RESULTS AND DISCUSSION

In this study, 7.0- and 6.4-log reductions of *E. coli* O157:H7 SEA13B88 and Oklahoma cells, respectively, were determined in 600-MPa–pressurized tomato juice at 35°C (data not shown). The use of HHP at low pH to inactivate *E. coli* O157:H7 in fruit juices and other fruit products has been reported *(1, 26)*. Patterson et al. *(32)* studied the sensitivity of vegetative pathogens including *E. coli* O157:H7 in buffer (pH 7.0), ultrahigh-temperature–processed milk, and poultry meat pressurized up to 700 MPa at 20°C. In that study, the authors achieved a 5-log reduction in the populations of all bacteria tested. In our study, the log reduction for both *E. coli* O157:H7 strains in tomato juice pressurized at 600 MPa at 25°C was higher than the 5-log reduction reported in apple juice *(16)* and orange juice (pH 3.9) pressurized at 550 MPa for 5 min at 20°C *(21)*. The higher (6- to 7-log) inactivation

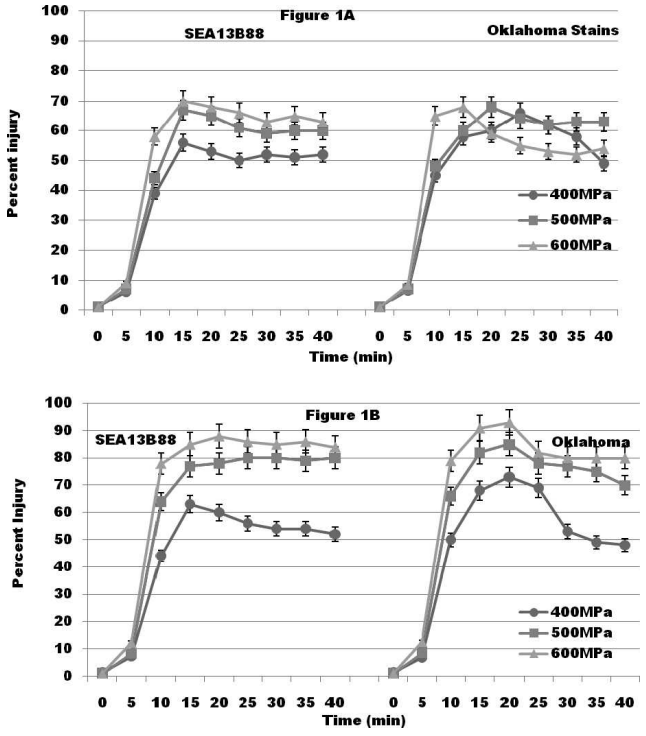

FIGURE 1. *Percent injury of* E. coli *O157:H7 SEA13B88 and* E. coli *O157:H7 Oklahoma in PBS pressurized at 25°C (A) and 35°C (B) for 40 min. Values are means of three determinations ± standard deviations.*

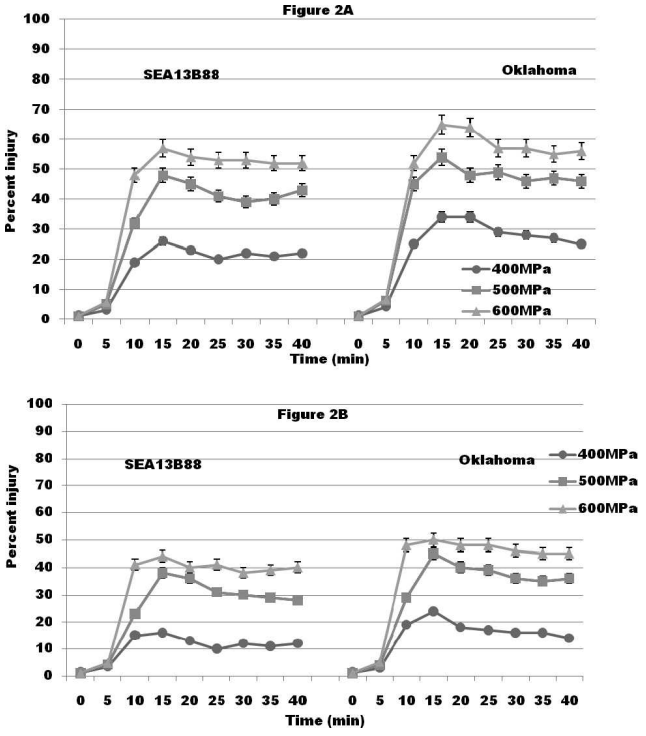

FIGURE 2. *Percent injury of* E. coli *O157:H7 SEA13B88 and* E. coli *O157:H7 Oklahoma in tomato juice pressurized at 25°C (A) and 35°C (B) for 40 min. Values are means of three determinations ± standard deviations.*

reported in our study could be attributed to the higher temperature (35°C) and a treatment time of 40 min.

Sublethal injury of bacterial cells in PBS and tomato juice. The percentages of populations of injured *E. coli* O157:H7 (Oklahoma) and *E. coli* O157:H7 (SEA13B88) cells in PBS pressurized at 25 and 35°C for up to 40 min are shown in panels A and B, respectively, of Figure 1. Higher populations of injured *E. coli* O157:H7 Oklahoma and SEA13B88 cells were determined at 15 min for all pressure treatments (400 to 600 MPa) at 25 and 35°C (Fig. 1A). Pressure treatment at 600 MPa for 15 min led to 70 and 68% of the *E. coli* O157:H7 (SEA13B88) and (Oklahoma) cells, respectively, being injured at 25°C. At 35°C and 600-MPa treatment, the injured populations increased to 84 and 90% in SEA13B88 and Oklahoma cells, respectively (Fig. 1B).

The results for injured *E. coli* O157:H7 SEA13B88 and *E. coli* O157:H7 Oklahoma populations determined in tomato juice pressurized at 25 and 35°C are shown in Figure 2. Pressure treatments at 25°C for 15 min caused approximately 58% of *E. coli* O157:H7 SEA13B88 cells being injured at 600 MPa, 45% at 500 MPa, and 22.6% at 400 MPa. The injured populations determined for Oklahoma cells in tomato juice pressurized at 25°C were 64% at 600 MPa, 53% at 500 MPa, and 33% at 400 MPa (Fig. 2A). Increasing the treatment temperature from 25 to 35°C for 15 min reduced the populations of injured *E. coli* O157:H7 cells (Fig. 2B). Pressure (600-MPa) treatment at 35°C for 15 min led to 44% of *E. coli* O157:H7 SEA13B88 and 50% of *E. coli* O157:H7 Oklahoma cells being injured in tomato juice. A significantly greater number of injured *E. coli* O157:H7 populations was determined in PBS (Fig. 1A and 1B) than in tomato juice (Fig. 2A and 2B), and *E. coli* O157:H7 Oklahoma had a higher population of injured cells than did *E. coli* O157:H7 SEA13B88 in pressurized tomato juice treated at 25 and 35°C for 40 min.

Leakage of intracellular substances of injured bacteria cells. The initial ATP levels in uninoculated, untreated PBS and tomato juice determined immediately before pressure treatments averaged 0.4 and 1.5 log fg/ml, respectively. These numbers represent extracellular ATP and are referred to as the baseline values. The initial extracellular ATP level determined in tomato juice was higher than the values in PBS (Fig. 3A and 3B). The ATP levels of PBS and tomato juice inoculated with *E. coli* O157:H7 bacteria and then pressurized (400 to 600 MPa) at 35°C for 30 min increased depending on the treatment time. In this study, we monitored bacterial ATP because ATP plays a key role in the energy status of the cell and in regulating enzyme activity *(11, 22)*; therefore, any perturbation that causes influx and/or efflux of substances in and out of the bacterial membrane may affect the energy status and the enzymatic activity of the cell, leading to its death. The pressure treatments tested in our study disrupted the *E. coli* O157:H7 membrane, and this led to increase in the extracellular ATP. The low level of ATP values in pressurized tomato juice could be attributed to a possible quenching activity of the bioluminescence process by the chemical attributes of tomato juice including the pH.

The leakage of intracellular UV materials of injured *E. coli* O157:H7 cells determined in 600-MPa–pressurized PBS

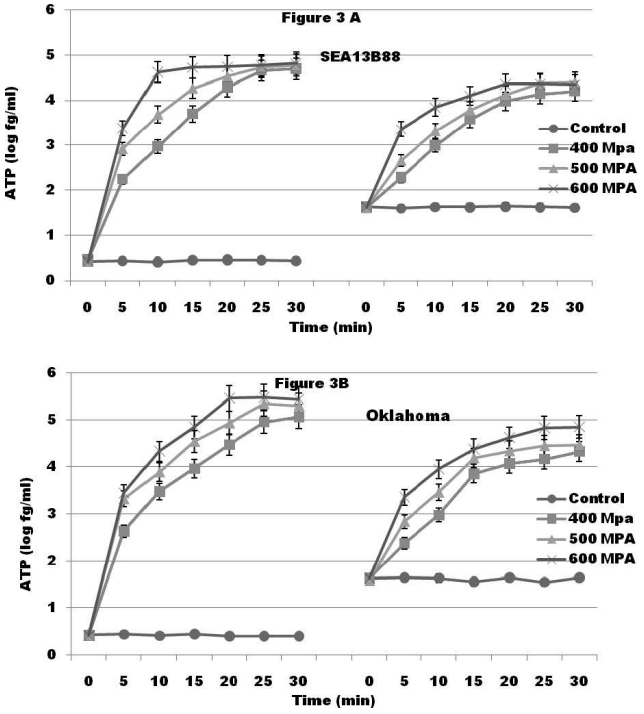

FIGURE 3. *Leakage of ATP from pressurized injured* E. coli *O157:H7 SEA13B88 (A)* and E. coli *O157:H7 Oklahoma (B) at 25 and 35°C for 40 min and its accumulation in treated samples. Values are means of three determinations ± standard deviations.*

at 25 and 35°C for 30 min is shown in Table 1. The initial UV materials of *E. coli* O157:H7 SEA13B88 in untreated PBS determined at A_{280} and A_{260} averaged 0.013 and 0.11, respectively. These values were not significantly ($P > 0.05$) different when inoculated with *E. coli* O157:H7 Oklahoma cells. The linear relationships between 600-MPa pressure treatments and treatment time at 25 and 35°C on cellular leakage of UV materials for each *E. coli* O157:H7 strain were not the same. For example, the UV materials of pressurized (25 and 35°C) injured *E. coli* O157:H7 Oklahoma cells determined at A_{260} had correlation values (R^2) of 0.9167 and 0.9066, respectively. The values determined for *E. coli* O157:H7 SEA13B88 similarly treated at A_{260} had R^2 of 0.9322 and 0.9288 at 25 and 35°C, respectively. When the linear correlations of UV materials of *E. coli* O157:H7 cells were determined at A_{280}, 600-MPa pressure treatment at 25 and 35°C led to an R^2 of 0.8892 at 25°C and an R^2 of 0.9259 at 35°C for *E. coli* O157:H7 Oklahoma cells and an R^2 of 0.8051 at 25°C and an R^2 of 0.8964 at 35°C for *E. coli* O157:H7 SEA13B88 cells. Pressure treatment at 600 MPa was chosen due to the fact that injured *E. coli* O157:H7 populations were higher than the values determined from the other pressures tested. The results of our study at A_{260} indicate more leakage of nucleic acid materials from *E. coli* O157:H7 SEA13B88 than *E. coli* O157:H7 Oklahoma. However, the reverse was the case for determinations made at A_{280} for all pressure treatments at 25 and 35°C for up to 35 min.

All pressure treatments tested in our study disrupted the *E. coli* O157:H7 membrane, and this led to an increase in UV materials in pressurized samples. The increase in extracellular ATP and the presence of UV materials

TABLE 1. *Comparison of linear regression and correlation coefficient of 600-MPa pressure treatments and temperature on leakage of UV materials of* E. coli *O157:H7*

	Regression coefficients[a]					
	25°C			35°C		
Absorbance	*a*	*b*	R^2	*a*	*b*	R^2
E. coli O157:H7 Oklahoma						
260 nm	0.130	0.0126	0.9167	0.1218	0.0152	0.9066
280 nm	0.0793	0.0210	0.8892	0.1061	0.0284	0.9259
E. coli O157:H7 SEA13B88						
260 nm	0.1232	0.0095	0.9322	0.1407	0.0141	0.9288
280 nm	0.0101	0.0167	0.8051	0.0929	0.0220	0.8964

[a] Linear regression coefficients were calculated based on the formula $Y = a + bx$, where b is the slope, a is the y-intercept, and R^2 is the correlation coefficient for each *E. coli* O157:H7 strain. Treatments were determined at 0, 5, 10 15, 20, 25, 30, 35, and 40 min.

determined at A_{260} (nucleic acid) and A_{280} (protein) in pressurized samples confirmed membrane damage for both *E. coli* O157:H7 strains. Other researchers have measured leakage of nucleic acids and proteins from microwave-injured bacteria and reported that the intracellular UV-absorbing substances that leaked out from the bacteria into the cellular extracts were mostly nucleic acids with some proteins *(31, 37, 39, 41)*. Also, leakage of bacterial intracellular substances as a result of membrane damage by antimicrobial agents has been reported *(2, 11, 22, 38)*. The authors of those studies concluded that the accumulation of extracellular ATP in media containing nisin, ampicillin, and streptomycin came from injured bacterial cells in the samples. The UV substances and ATP that leaked out from the injured bacteria cells diminished the capacity of the *E. coli* bacteria to maintain normal physiological activity, which ultimately led to their death.

Effects of storage on injured populations. The effects of storage temperature on injured populations of resting *E. coli* O157:H7 SEA13B88 and Oklahoma cells in PBS pressurized at 400, 500, and 600 MPa at 25 and 35°C determined during storage at 23°C for up to 1,800 min are shown in Figure 4A and 4B. The initial populations of injured *E. coli* O157:H7 SEA13B88 cells surviving 400- and 500-MPa treatments at 25°C for 25 min in PBS remained the same for up to 360 and 720 min in samples treated at 600 MPa and stored at 23°C. At 720 min of storage, the population of injured *E. coli* O157:H7 SEA13B88 cells determined in 400- and 500-MPa–pressurized PBS at 25°C increased from 6 log to approximately 7 log at 1,800 min of storage. In PBS pressurized at 600 MPa (25°C), the surviving population increased from 5 to 6 log at the end of storage (1,800 min). In PBS pressurized at 35°C and stored at 23°C for 1,800 min, the surviving populations increased but were <0.6 log CFU/ml at 1,800 min depending on the amount of pressure used (Fig. 4A). The data determined for resting *E.*

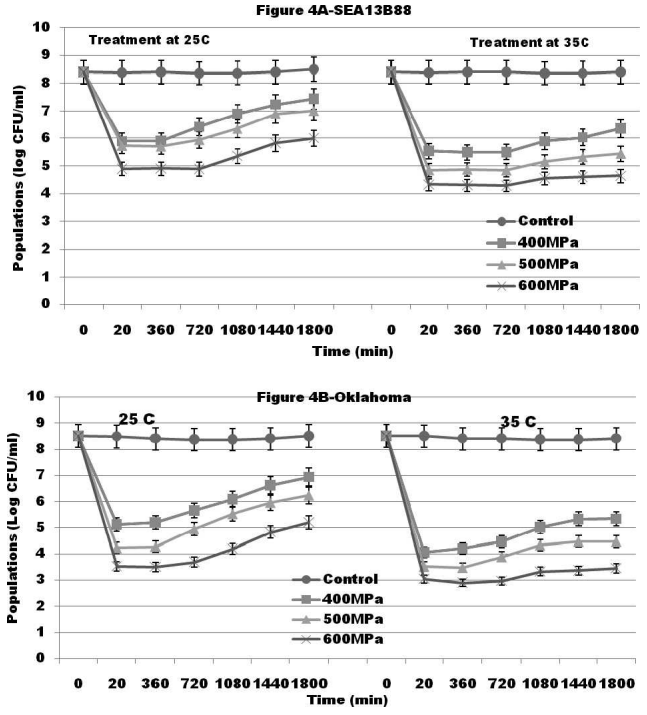

FIGURE 4. *Survival of pressurized injured* E. coli *O157:H7 SEA13B88 (A)* and E. coli *O157:H7 Oklahoma (B) at 25 and 35°C for 40 min in PBS during storage at 23°C. Values are means of three determinations ± standard deviations.*

coli O157:H7 Oklahoma cells in PBS similarly treated and stored at 23°C for 1,800 min are shown in Figure 4B. Again, pressure treatments at 35°C led to lower populations of surviving *E. coli* O157:H7 Oklahoma cells than treatment at 25°C, suggesting that *E. coli* O157:H7 Oklahoma cells appeared to be more susceptible to pressure treatment at 35°C than at 25°C.

In tomato juice, the initial populations of *E. coli* O157:H7 SEA13B88 and Oklahoma cells in samples pressurized at 25 and 35°C declined during storage at 23°C for 1,800 min (data not shown). Pressure treatment at 35°C and storage at 5°C decreased the surviving injured population to below detection at 720 min in tomato juice treated at 500 and 600 MPa and 1,080 min in tomato juice treated at 400 MPa. In all, more recovery of injured populations of *E. coli* O157:H7 Oklahoma and SEA13B88 was observed in PBS than in tomato juice irrespective of the pressure treatments and temperature used. Unlike what was observed with storage of pressurized PBS samples at 23°C for 1,800 min, pressurized tomato juice stored at 5°C for 1,800 min did not allow recovery of injured bacterial cells.

Other researchers have reported similar findings in fruit juices *(16)* and PBS *(24)*. In our study, we extended the storage time to 1,800 min (30 h), and the population of the surviving *E. coli* O157:H7 cells was below detection (<2 CFU/ml) in pressurized tomato juice stored at 5°C. It is true that there may be viable but nonculturable HHP-injured cells in the samples; our attention was focused only on viable but culturable injured *E. coli* cells. However, cold storage of pressurized PBS and tomato juice at 5°C for 1,800 min led to further decline of injured surviving *E. coli* O157:H7 cells treated at 25 and 35°C (data not shown). It is our conclusion that *E. coli* O157:H7 SEA13B88 and Oklahoma cells reacted differently to HHP treatment at 25 and 35°C as indicated by leakage of intracellular UV materials and ATP of injured bacteria. The inability of the injured bacteria to repair could be attributed to the loss of intracellular UV materials and ATP, and the cold (5°C) temperature storage also contributed to further inactivation of the injured populations.

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